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IMPROVEMENTS IN THE PARAFFIN METHOD

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ABSTRACT.—**Dilute hydrofluoric acid alone and in conjunction with glycerin and ethyl alcohol was employed successfully to soften various types of refractory plant materials embedded in paraffin. Serial sections were cut at 6–8 μ and no appreciable deleterious effects on cell walls, cell contents, or staining procedures occurred. "Tannins" and "phlobaphene compounds" can be removed from tissues softened by this method by treating the sections for 12–48 hours with an aqueous solution of chromic acid, potassium bichromate and glacial acetic acid prepared according to the formula given by Johansen (1940).**

I. SOFTENING OF REFRACTORY PLANT MATERIAL EMBEDDED IN PARAFFIN

Certain plant materials, as well as animal tissues, tend to be extremely difficult to section after being embedded in paraffin. Methods utilizing mixtures of glycerin-alcohol and glycerin-anilin-oil, have already been recommended for softening embedded animal tissues (Baker, 1941; Lendrum, 1944). In a modified form these methods can also be adopted for plant tissues. Exposing embedded plant material to warm water, as suggested by Johansen (1940), Sass (1940), and Ball (1941), has also been advocated but this technic may result in the serious maceration of tissues, especially if the treatment is prolonged.

It has been assumed rather generally that difficulty in sectioning such material as woody stems, coriaceous leaves, roots, etc., was produced by the lignin and silica in the walls of fibers, sclereids, and tracheary elements. Kerr (1934), however, has shown that failure to microtome woody materials successfully is due largely to the condition of the cellulose in the walls rather than to their mineral and lignin content. According to Kerr, the softening action of hydro-

fluoric acid on woody tissues "is associated with a partial degradation of cellulose and the formation of hydrocellulose". This results in a change "in the physical properties of the fibers".

In the light of the above methods, the writers have tested the softening action of hydrofluoric acid, either alone or in conjunction with glycerin-alcohol, on a wide range of plant tissues after embedding in paraffin. Specimen vials lined with paraffin were used and prior to treatment a small area of the embedded tissue was exposed by trimming with a razor blade. All materials used in these experiments were embedded in "Tissuemat". The composition of the various solutions and the exposure times are presented in the following table.

TABLE 1. COMPOSITION OF FLUIDS AND EXPOSURE TIMES.

Schedule	Composition	Exposure-period Room Temp. 22°C.±
1.	10–15% hydrofluoric acid	3 to 7 days
2.	20 ml. glycerin 80 ml. of 70% ethyl alcohol followed by: 10% hydrofluoric acid	1 week 3 to 7 days
3.	10 ml. glycerin 10 ml. hydrofluoric acid 80 ml. 95% ethyl alcohol	3 days to 2 weeks

The exposure times listed in the above table proved satisfactory for the authors' materials but doubtless will vary depending upon the "hardness" of the tissues in each case. The softening action of the reagents may be accelerated by placing the specimen vials in a 32° C. electric oven. Tests with a sharp razor blade should be made at intervals until the desired texture is obtained.

Any of the above schedules appreciably softened the materials investigated but schedules 2 and 3 usually yielded smoother ribbons, apparently because of the "lubricating" action of the glycerin. Material allowed to remain in aqueous hydrofluoric acid for long periods may show signs of maceration. This may be alleviated by the use of ethyl alcohol. Schedule 3 is recommended for general use. It has been employed successfully in microtoming such material as the coriaceous leaves of *Mouriria Huberi* Cogn. (Foster, 1947) and the scaly winter buds of species of *Drimys*, *Pseudowintera*, *Illicium* and *Trochodendron*.

This procedure has been followed by successful staining. The writers have followed the tannic acid, iron chloride, safranin schedule of Foster (1934). In addition, the common fast-green-safranin

schedule is not altered. No visible alterations or deleterious effects of these treatments have been observed in the plant tissues so treated.

II. The Removal of Tannins and Phlobaphene Compounds from Paraffin Sections

Often associated with processed material which is difficult to section is the presence of tannins and phlobaphene compounds. This is particularly true of many tissues in bud scales, leaves, stems and roots. Johansen (1940) has described a method, originated by Dr. Palmer Stockwell, of treating paraffin sections prior to staining. The formula is as follows:

Chromic acid	1 g.
Potassium biochromate	1 g.
Glacial acetic acid	10 ml.
Distilled H_2O	90 ml.

Depending on the material in question, it has been found that 12 to 48 hours is required to remove adequately the tannin compounds. Slides should then be washed in running water for 1 to 2 hours before proceeding to the usual staining schedules.

This procedure is of great value in histogenetic studies since there is a tendency for the embryonic layers of certain leaves, bud scales, and young stems to accumulate "tannins" to the point of obscuring nuclear divisions and plastid structure. Following this treatment, staining is noticeably improved. If the maximum time of treatment is required, there is a tendency for the sections to drop off unless the adhesive described by Sass (1940 p. 50–51, Formula III) is used.

The potency of the bleaching fluid decreases with age so that a fresh solution should be prepared after marked oxidation has occurred.

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THE STABILITY OF SO-CALLED AXONAL ACID PHOSPHATASE AS DETERMINED BY EXPERIMENTS IN ITS "STAINABILITY"

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Abstract.—**Since the histochemical method for exhibiting acid phosphatase in bodily tissues is said to depend upon the enzyme acting on suitable substrates, it is possible to test its stability by various tests. It has been found that the background element or elements, whatever they may be, concerned with the "staining" properties of the reaction are very stable and somewhat resistant to destruction. So-called acid phosphatase in spinal cord axons has not been inactivated by subjecting it to various fixing solutions, changes in temperature and pH, relatively prolonged post-mortem autolysis nor by well known enzyme inactivators. It is believed that the "staining" reaction may be dependent fundamentally on other factors than enzymatic activity.**

Since Gomori (1941) introduced his histochemical method for detecting acid phosphatase in bodily tissues, an increasing number of investigators have utilized this approach on normal or pathological tissues of different animals. A number of speculations have been made concerning its possible functional and pathological significance. The method is said to depend on the action of the enzyme, when present in tissues, to split the phosphate radical from a suitable substrate (sodium glycerophosphate and others) contained in a solution having an acid buffer mechanism and lead nitrate. The hydrolyzed phosphate ion is supposed to combine with lead to form lead phosphate at the exact site of enzymatic activity. Subsequent treatment with ammonium sulfide converts any lead phosphate formed to lead sulfide which has a distinct brown coloration. Acid phosphatase, like other enzymes, is thought to be very sensitive to temperature and pH changes and certain other inactivating substances. In regenerative studies of the sciatic nerve in the cat, it was noticed that this so-called enzyme "stained" following injection of 40% formalin into the nerve with subsequent immersion in acetone; as a result, its stability was tested further by other experiments.

[1]With the assistance of Miss Margaret Powers, technician.

Methods

A list of the methods used to test the durability of so-called acid phosphatase in axons of the brain stem and spinal cord of cat, monkey and man is given below:

1. The effect of fixatives:
   (a) 10% formalin—fresh tissues fixed for three days.
   (b) 40% formalin (commercial)—fixation time, one day.
   (c) Protargol fixative—butyl alcohol, 65 ml.; propyl alcohol, 25 ml.; formic acid, 5 ml.; and trichloracetic acid, 5 ml. Fixation time, three days.
   (d) Ranson's pyridine silver fixative—absolute alcohol, 98 ml.; and ammonium hydroxide, 2 ml. Fixation time, two days.

2. The effect of immersion of acetone-fixed sections in fluids with extreme pH ranges.
   (a) 1% hydrochloric acid—one hour.
   (b) 1% nitric acid—one hour.
   (c) 1% ammonium hydroxide—one hour.

3. The effect of temperature.
   (a) Heating fresh tissue in warmed acetone (39° C.)—for ninety-six hours.
   (b) Heating fresh cord tissue in water at 65° C. for periods up to three hours, then fixation in cold acetone.
   (c) Incubation of acetone-fixed sections at 65° C. instead of 37° —for periods varying from one to twenty hours.
   (d) Boiling acetone-fixed sections in water—for one hour.
   (e) Refrigeration of fresh tissue at 4° C.—for various intervals up to thirteen days followed by fixation in cold acetone.
   (f) Effect of room temperature on fresh unfixed tissue—up to forty-eight hours followed by fixation in cold acetone.

4. Effect of enzymatic inactivating substances.
   (a) Immersion of acetone-fixed sections in 10% hydrogen peroxide solution—for one hour.
   (b) Immersion of acetone-fixed sections in 10% solution of phosphotungstic acid—for one hour.
   (c) Immersion of acetone-fixed sections in 10% solution of mercuric chloride—for one hour.
   (d) Immersion of acetone-fixed sections in 1% solution of picric acid—for one hour.

The methods used for "staining" the so-called enzyme were two in number: Gomori's original incubating mixture and one which employ-

ed ascorbic acid as an additional compound. The exact procedure of each is listed below:

Gomori's Method.

Molar acetate buffer pH 5	30 ml.
Aqueous lead nitrate (5%)	10 ml.
Distilled water	60 ml.
Alpha sodium glycerophosphate (52%) in 2% aqueous solution	30 ml.

Ascorbic Acid Solution.

Molar acetate buffer, pH 4.7	10 ml.
Aqueous lead nitrate (5%)	4 ml.
Distilled water	60 ml.
Alpha sodium glycerophosphate (52%) in 2% aqueous solution	12 ml.

Just before incubation, 2 ml. of ascorbic acid (Cevalin, Lilly) is added to the above solution.

The normal incubation temperature for both of these methods is 37° C. The incubation times used varied from one to ninety-six hours.

Results

The results of the study indicate that the substance which is "stained" in the axis cylinders of the central nervous system by the two histochemical technics for exhibiting acid phosphatase is remarkably stable and somewhat resistant to destruction. None of the methods employed to test its stability have completely inactivated it.

Positive presence of the so-called enzyme has been obtained following fixation with 10 and 40% formalin (Fig. 2), Ranson's fixative, and that which is used in the protargol technic.

Immersion in fluids having extreme pH ranges, such as 1% ammonium hydroxide, 1% nitric acid, or 1% hydrochloric acid for one hour, did not destroy or inactivate the so-called enzyme (Fig. 3 and 4).

The "stainable" substance also withstood ranges in temperatures well. Positive results were obtained after heating fresh cord tissue for ninety-six hours in warm acetone at 39° C.; heating fresh, unfixed cord tissue in water at 65° C. for three hours (Fig. 6); incubating acetone-fixed sections at 65° C. and boiling acetone-fixed sections containing the so-called enzyme for one hour (Fig. 1). In an animal which was kept in a refrigerated ice box, having the constant temperature of 4° C., the "stainable" substance was partly preserved up to thirteen days. Very slight breakdown began in the axis cylinders of

the cord after twenty-four hours, especially in the largest axis cylinders, and gradually continued. The changes were more rapid in the specimen left at room temperature; the so-called enzyme was considerably broken down after twenty-four (Fig. 5); at forty-eight hours, a few axons still "stained". In all of the cases studied the axons located in the posterior funiculus seemed to be more resistant to temperature changes than in other portions of the cord.

Immersion in such well known enzymatic inactivating solutions as hydrogen peroxide, phosphotungstic acid, picric acid or mercuric chloride did not affect the "stainable" substance to any material degree. If anything, they actually seemed to enhance the intensity of the "staining".

The addition of ascorbic acid or vitamin C (Cevalin, Lilly) to the incubating mixture seemed to speed up or vitalize the reaction considerably. A positive reaction was obtained by using ascorbic acid alone without the use of sodium glycerophosphate or any other substrate. With the addition of ascorbic acid to the incubating solution, it took on the average, about one-fifth as long as Gomori's method to exhibit adequately the "stainable" substance.

Comments

Most enzymes are easily affected by changes in temperature, hydrogen-ion concentration, heavy metals, phosphotungstic and picric acids and oxidizing agents. Some of the common solvents for enzymes are water, dilute ethyl alcohol and dilute acids and alkalis.

EXPLANATION OF FIGURES

Photomicrographs taken under high power showing the presence of so-called acid phosphatase in spinal cord axons of cat following various experimental procedures. All sections incubated at 37° C.

1. Following sixty minutes boiling in water of acetone-fixed sections—21 hours incubation in ascorbic acid solution.

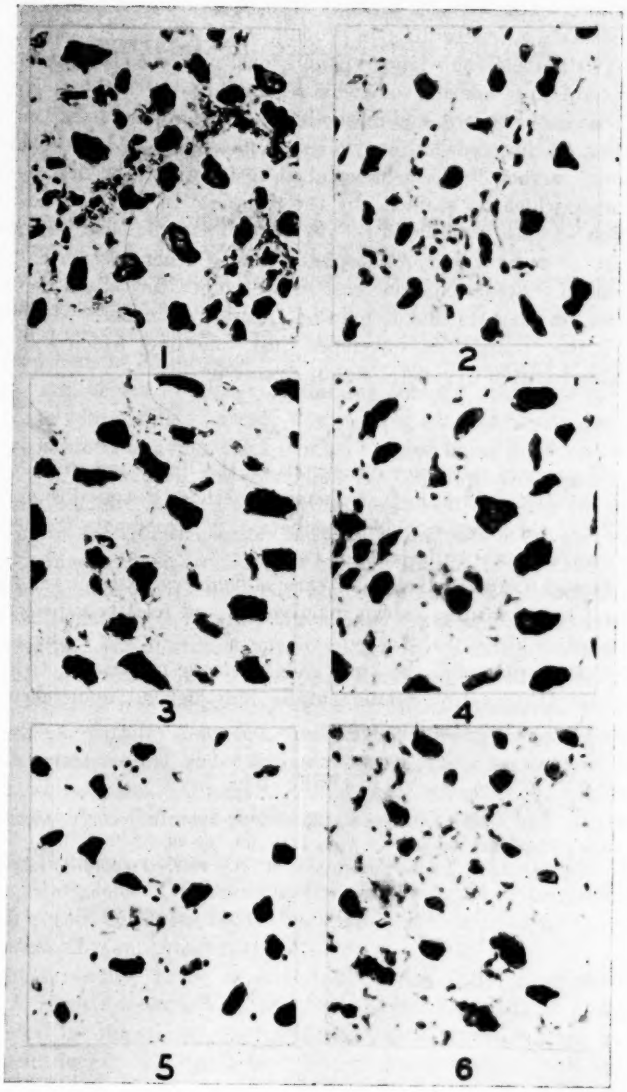
2. Following fixation in 40% (commercial) formalin—96 hours incubation in Gomori's solution.

3. Following immersion of acetone-fixed sections in 1% ammonium hydroxide for one hour—24 hours incubation in ascorbic acid solution.

4. Following immersion of acetone-fixed sections in 1% nitric acid for one hour—24 hours incubation in ascorbic acid solution.

5. Following exposure of spinal cord to room temperatures (65° F.) for twenty-four hours—acetone-fixed sections incubated for 24 hours in Gomori's solution.

6. Following three hours heating of fresh cord tissue in water at 65° C., then fixation with acetone—incubation for 5 hours in ascorbic acid solution.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Hawk, Oser and Summerson (1947) state that the majority of enzymes are inactivated by heating them in solution to about 60° C. This involves a denaturation of protein.

The "stainable" substance in the axons, whatever it is, seems to be exceedingly stable and somewhat resistant to destruction. Nearly all of the above criteria and methods have been applied to testing the stability of the so-called enzyme acid phosphatase, by the histochemical method. These have failed to inactivate or destroy the substance which is "stained" by the technic. In several trials, the reaction was also positive in the presence of ascorbic acid but in the absence of sodium glycerophosphate or any other substrate. The response of the reaction to increase in the temperature of the incubating solution suggests that it may be chemical in nature. If this is true, it may possibly be developed into something which will be a valuable addition to general staining procedures.

Conclusions

1. The axonal substance in neurons of the brain and spinal cord, which is "stained" by the histochemical methods designed to exhibit activity of the enzyme, acid phosphatase, is exceedingly stable and somewhat resistant to destruction.

2. It withstands the action of various fixatives, extreme ranges in pH and temperatures, known inactivators and relatively prolonged post mortem autolytic activity.

3. Certain phases of the investigation on the material tested suggest that the reaction fundamentally may depend upon chemical rather than enzymatic phenomena.

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DIFFERENTIAL STAINING OF TISSUE IN THE BLOCK WITH PICRIC ACID AND THE FEULGEN REACTION

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ABSTRACT.—**The authors have found a modification of the Feulgen reaction to be a satisfactory stain for tissue in the block.**

Pieces of fresh mammalian tissue not thicker than 5 mm. are fixed for approximately 48 hours at 25° C. in a mixture of equal parts of 5% aqueous sulfosalicylic acid and saturated aqueous picric acid. They are washed for 30 minutes in three ten-minute changes of distilled water and placed in Feulgen's staining solution diluted to one-half strength with distilled water. The staining solution is allowed to act for 24 hours (2 to 3 mm. thick blocks) up to 48 hours for 5 mm. thickness. After staining, the specimens are transferred to a mixture of sodium bisulfite, 0.5 g. and *N* hydrochloric acid, 5 ml. in 100 ml. of distilled water. Two changes of 15 to 30 min. each in the acid sulfite are given and these are followed by dehydration through 50%, 70% and 95% alcohol. One to two hours are allowed for each change except the last 95%, in which the stained tissue is allowed to remain overnight. The dehydration is completed in two changes of absolute alcohol with subsequent clearing in xylene and embedding in paraffin. Sections may be cut 10 μ or other thickness desired, mounted on slides, paraffin removed, and covered in the usual manner. Nuclei stain reddish violet against a lemon yellow background when the stain is typical. Orange G, 200 mg. per 100 ml. may be added to the fixing fluid if a more polychromatic effect is desired.

The Feulgen reaction is employed usually to stain sections. However, Margolena (1931) used it for whole-mounts of protozoa and small worms, and Liang (1947) has adapted it to staining nerve trunks in blocks of mammalian tissue.

The procedure to be described developed from experiments in which Liang's technic was used both in its original form and with essential modifications. In his method, fresh tissue is fixed in 1% aqueous formic acid for 1 to 3 hours, rinsed, stained with Schiff's reagent until the nerves appear, washed about an hour in a solution of

[1]Contribution No. 483.

sulfurous acid, and finally dehydrated. Whole-mounts of thin material can be made or the blocks can be sectioned after embedding in paraffin. We found the method to be very reliable for bringing out the topography of small peripheral nerve trunks which contained myelinated fibers. The leuco basic fuchsin stains myelin readily under the conditions listed above. We were unable, however, to secure staining of unmyelinated fibers.

Prolongation of the time of fixation in acid tends to bring in the staining of cell nuclei and therefore produces the typical "nucleal" reaction described originally by Feulgen and Rossenbeck (1924). This fact, coupled with the observation that the presence of picric acid used in fixation did not interfere with the staining of nuclei, suggested that a technic for block staining in two colors might be developed.

Material and Methods.

Fresh tissues from rat, kitten, rabbit, dog and monkey were fixed for periods of time varying from 1.5 hours to 8 days. Spleen, intestine, intercostal muscle, trachea, esophagus, thyroid gland, spinal cord or brain, and sciatic nerve were used to give a wide variety of tissues and structures. The facility of penetration of both the picric acid used in the fixative and the subsequent Feulgen reaction was tested with pieces of spleen which measured about 1 cm. through their thinnest dimension.

Only a limited number of fixing fluids were tried because it is essential for best results that the hydrolytic effect of the acid used in fixation be adequate to bring out the Feulgen reaction and yet not so vigorous that inadequate time will be allowed for good penetration of the picric acid. Since the objective of the technic was to secure a bicolor or polychromatic stain in the tissue block, picric acid served both as a background stain and fixing agent. Trichloracetic acid and sulfosalicylic acids were the logical ones to use as hydrolytic agents. Both are good protein precipitants and penetrate rapidly. They are rather highly ionized, hence strong organic acids.

The two fixing fluids used most were the following:

Sulfosalicylic acid, 5% aq	50 ml.
Picric acid, sat. aq	50 ml.
and	
Trichloracetic acid, 5% aq	50 ml.
Picric acid, sat. aq	50 ml.

In some experiments, formalin to make 5% of the volume of the acid mixture was added. When formalin was used a very thorough wash-

ing was given the tissue after fixation to remove it before placing the tissue in the staining solution. (See Bauer, 1932, on the use of formalin-fixed tissues for the Feulgen stain.) The addition of formalin seemed to improve the fixation somewhat, but its effect was not specially marked in conjunction with the rather efficient acid mixtures. Of the two acids, sulfosalicylic seems preferable to trichloracetic since it gave more consistently good fixation and subsequent staining.

Attempts to introduce a third color into the final stain by adding either aniline blue or fast green FCF to the fixing solution were unsuccessful, since neither of these dyes penetrated the tissue for more than about 0.5 mm. However, when orange G was added to the fixative in a concentration of 0.2%, its ability to penetrate appeared to be about the same as that of picric acid.

The Schiff's reagent used for staining was not varied and was prepared in the manner recommended originally by Feulgen. Advantage was taken of the improvements suggested by de Tomasi (1936) and the method for removal of residual color with activated carbon by Coleman (1938.) The stock solution was usually diluted with an equal volume of distilled water just before use. The time of staining was varied as follows: 6, 12, 24, 48 and 72 hours. The results obtained indicated that penetration of the stain was rather slow and that there was no serious tendency toward overstaining. Hence the optimum time is not very critical but should be sufficient for complete penetration. The best results in our series appeared in the 24 and 48 hour stains. Blocks more than 5 mm. thick required at least 48 hours and spleen was frequently not fully penetrated even then. The 72-hour specimens were less well differentiated and somewhat overstained. Small blocks, such as 6 to 8 mm. lengths of rat intestine, were usually stained adequately in 24 hours.

After staining, the blocks of tissue were either treated for 0.5 to 1.0 hour in a dilute solution of sulfurous acid (formula in step 4 of method recommended below) or washed directly in water. We have gained the impression that the staining is sharper when the sulfurous acid is used between the staining bath and the final washing, which is in agreement with the recommendations given in the literature for staining sections by the Feulgen technic.

Any residues of staining solution or sulfite left in the tissue after treatment with sulfurous acid appear to be soluble in 50% alcohol, hence washing in water after this step was usually omitted, although washing in distilled water did no harm. Alkaline tap water would tend to remove the picric acid stain, likewise alkalinity would be undesirable in the alcohol used for dehydration.

The results obtained from 400 blocks of tissue processed to include the variables listed above indicate that the following technic can be recommended:

1. Fix fresh animal tissue in the following mixture for not less than 36 nor more than 60 hours at room temperature (25°C.).

Sulfosalicylic acid	2.5 g.
Distilled water	50 ml.
When dissolved, add	
Picric acid, sat. aq	50 ml.
Orange G (optional)	100 mg.

2. Wash through 3 changes of distilled water, 10 min. each, to remove the excess of the fixing fluid.

3. Place in Feulgen's staining solution (Schiff's reagent) diluted with distilled water to one-half the strength of the stock solution. The stock solution is prepared as follows:

Dissolve 0.5 g. basic fuchsin by pouring over it 100 ml. boiling distilled water. Shake thoroughly and cool to 50°C. Filter and add 10 ml. 1*N* HCl to filtrate. Add 0.5 g. $K_2S_2O_5$ (potassium metabisulfite) and allow to stand in the dark well stoppered for 18 hours or until it is a light straw color. To remove residual color add 0.25 g. of activated charcoal, shake for a minute or two and filter. If the solution is not clear, refilter through the charcoal. Keep the bottle well stoppered at all times.

Stain for 24 hours if blocks are small, 2–3 mm. thickness, or 48 hours for 5–7 mm. Limit thickness to 5 mm. if possible. Both fixing and staining are carried out at room temperature (about 25°C.).

4. Transfer the specimens to sulfurous acid solution:

Sodium or potassium bisulfite	.5 g.
Normal hydrochloric acid	5 ml.
Water	100 ml.

Use two changes of 15–30 min. each.

5. Dehydrate through 50%, 70% and 95% alcohol, 1–2 hours each, allow to remain in a second change of 95% overnight and follow with two changes of absolute alcohol and two of xylene.

6. Embed in paraffin.

7. Cut sections 8 to 15μ thick, mount, remove paraffin with xylene and cover in the usual manner.

Discussion

The stain shows nuclei in hues of reddish violet against a lemon yellow background when orange G has not been added to the fixing fluid. When orange G was added, red blood cells and voluntary muscle usually took it up and added thereby some degree of differen-

tiation among the nonnuclear tissue components. The shafts of hairs always stained bright greenish yellow. Epidermis and white fibrous connective tissue were pale yellow to lemon yellow. Smooth muscle was stained by the picric acid but the sarcoplasm of striped muscle stained orange. In some fibers the anisotropic bands were seen to be more darkly stained than the isotropic but the finer H and Z lines were not distinguishable. The exact hues of the polychromatic effects obtained appeared to be somewhat unpredictable, but they permitted the different tissue components to be distinguished easily.

Structures other than nuclei were stained by the Feulgen reaction. The submucosa of intestine, cartilage, the intima of blood vessels and, occasionally, the fibrils of elastic tissue stained violet. Myelin stained violet to reddish violet and always stained whether the period of fixation was 1.5 hours or 48 hours. Elastic tissue was more likely to stain after a short period of fixation but not after a long one. The nuclei of liver and striped muscle did not stain as strongly as those of tubular glands and epithelium.

As given, this method does not produce pleasing results when used with tissue of the central nervous system. Although color differentiation exists between the grey and white matter, the details of the tissues are not sufficiently defined for the usual purposes of the neurohistologist.

The ease of execution and reliability of this technic adapts it to uses which require good differentiation of nuclei in serial sections and classroom slides. Since the staining is completed in the block, the method can be substituted for hematoxylin and eosin with considerable saving of time. Like most methods, it is neither perfect nor infallible. Lack of penetration of the Schiff's reagent limits the usable thickness of the tissue to about 5 mm. Blocks of greater width and length can be used provided that the pieces are made rather thin.

The permanence of the stain has been tested only to the extent of leaving slides in direct sunlight for approximately 50 hours total exposure. The amount of fading was not noticeable when the preparations were compared with unexposed slides.

Plant material that remained in the lumen of the intestine showed typical nuclear staining.

The fact that nucleoproteins are undergoing hydrolysis during fixation, which is one of the essentials of the Feulgen reaction, makes a time schedule for the entire process necessary. This time schedule is not rigid, but gross variations from the 48-hour fixing time are undesirable. In the regular hydrolysis for the Feulgen stain with sections, the time is approximately 0.1 hour in *N* HCl at 60°C. In

the block staining process described, hydrolysis occurs at about 25° in an acidity that is slightly less than 0.1 *N*. If the theoretical time needed for hydrolysis in the latter process be calculated by Vant Hoff's law for temperature effects, and the law of squares for ionic activity, the following equations are used.

(1) 60°–25°=35° difference in temperature. If the reaction time doubled for each 10°, the reaction rate at room temperature should have been $\frac{1}{3.5 \text{ squared}}$ or approximately $\frac{1}{12}$ that at 60°.

(2) The reciprocal of the square of the ionic concentration of H^+ is roughly $\frac{1}{100}$. Hence the theoretical rate in the block is about $\frac{1}{1200}$ that of the customary one for hydrolyzing sections.

(3) Sections are hydrolyzed about 0.1 hour so that theory would require a period of hydrolysis in our technic with blocks of 1200 × 0.1 or 120 hours.

Experiments indicate that 120 hours is too long and that the optimum is somewhere near 48, therefore it appears that the hydrolysis does not follow strictly the above laws, or that the presence of picric acid influences the liberation of bonds in nucleoproteins and thus activates them to produce the characteristic Feulgen stain.

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SOME OIL SOLUBLE DYES WHICH STAIN SUBERIZED DEPOSITS IN ORANGE VESICLES[1]

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ABSTRACT.—**Of 16 commercial oil-soluble dyes, not previously in common use among botanists, eight (including oil blue NA, already known as a stain for rubber) have proved better than Sudan III and Sudan IV in staining suberized deposits in orange vesicles.**

Supplementary to a chemical analysis of the "fatty" constituents of citrus fruit is a study of the "fat" staining tissues of the fruit. In the course of this study it was found that the fruit vesicles had peripherally located deposits of suberin which were effectively stained by either Sudan III or IV as shown in Fig. 1. Other tissue substances

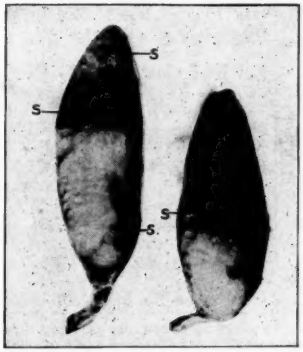

FIG. 1.—Suberized deposits, S, of orange vesicles, × 5.

listed as having affinity for these dyes include fats or oils contained singly in tissues or as components of complex tissue impregnations as is the case for suberin and also cutin and resin (Johansen, 1940). The same dyes are also employed to stain rubber in plant tissues (Haasis, 1944).

Recently, however, a commercially used oil soluble dye, oil blue NA, was found effective in staining rubber deposits in guayule (Addicott, 1944; Whittenberger, 1944) as well as the other tissue substances

[1]Agricultural Chemical Research Division Contribution No. 194.

listed above as having affinity for the Sudan dyes. Subsequently, this same dye, oil blue NA was found applicable as a fat stain in animal tissues (Lillie, 1945). In the case of the suberized areas of citrus vesicles, oil blue NA gave a stain which was exceedingly more brilliant than that achieved with either Sudan III or IV. Consequently, numerous other commercially recommended oil soluble dyes were tried as suberin stains using the same fruit material, with the possibility of their having further use comparable to that of Sudan III and IV.

TABLE 1. OIL SOLUBLE DYES FOR USE AS FAT STAINS.

Common Name	Federal Name	CI No. or Identity
Oil red OS (CCC)	D&C red No. 18	No CI No. 1-xylylazoxylylazo-2-naphthol
Oil red OB (AACC)	————	Technical dye Sample R-6033
Oil red XO (CCC)	FD&C red No. 32	No. CI No. 1-xylylazo-2-naphthol
Calco oil red (CCC)	————	Sample No. 1700
Rhodamine B stearate (CCC)	FD&C red No. 37	No CI No.
Alizarine irisol base (CCC)	D&C blue No. 3 and D&C violet No. 2	CI-1073
Calcogas blue NA or Oil blue NA (CCC)	Ext. D&C blue No. 5	No CI No. 1,4-bis-amino-anthraquinone
Spirit Blue (NACC)	————	CI No. 689
Oil orange SS (CCC)	FD&C orange No. 2	No CI No. 1-0-tolylazo-2-naphthol
Orange TR (CCC)	D&C orange No. 14	No CI No. 4,5,15-tribromo-2-7-dicarboxy-3,6-fluorandiol
Oil yellow AB (CCC)	FD&C yellow No. 3	CI-22
Oil yellow OB (CCC)	FD&C yellow No. 4	CI-61
Quinoline yellow—spirit soluble (CCC)	D&C yellow No. 11	CI-800
Alizarine cyanine green base (CCC)	D&C green No. 6	CI-1078

The dyes were supplied through the courtesy of the sources indicated as (CCC) = Calco Chemical Company; (AACC) = American Aniline and Chemical Company, and (NACC) = National Aniline and Chemical Division, Allied C.&D. Corp.

A staining technic found convenient was similar to that recommended for the treatment of animal tissues with fat stains (Lillie, 1944). Accordingly, the dyes were used in a 30:30:40 mixture of ethyl alcohol, acetone and distilled water, containing about 50 mg. of dye per 100 ml. of solvent. All dye solutions were filtered before using. Prior to staining, the fresh vesicles were immersed in the solvent mixture for a few seconds to prevent later precipitation of the dye in the tissue. Staining was for five minutes. After staining, the vesicles were again immersed in the solvent mixture, in 50% ethyl alcohol and finally in distilled water.

The dyes found effective in staining the suberized areas of the fruit vesicles, including oil blue NA, are listed in Table 1. Not only oil

blue NA, but also oil red XO, oil red OB, Calco oil red, alizarine irisol base, spirit blue, Calco orange SS and oil yellow AB stained the suberized areas more brilliantly and more deeply than did the Sudan dyes.

It was considered significant to record these observations for application in similar studies concerned with the demarcation of various plant substances. Frequently for such analysis, it is advantageous to have available a dye of a certain color for contrast with that of tissue areas surrounding a particular substance, and generally brilliance in staining facilitates such microscopic study.

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PHLOXINE AS AN HISTOLOGIC STAIN, ESPECIALLY IN COMBINATION WITH HEMATOXYLIN

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ABSTRACT.—**A staining schedule employing phloxine as a counterstain to Erlich's acid hematoxylin is presented. Fixation is best with Zenker's fluid, although formalin can be used. The technic is similar to the standard hematoxylin-eosin formulae but because of the staining advantages of phloxine over eosin, the technic is simpler, and quicker, resulting in clearly differentiated sections which do not fade as soon as do eosin-stained slides. A brief summary of the uses of phloxine as a biological stain is given and its advantages over eosin are discussed.**

INTRODUCTION

For some time the standard staining technics for histology have included variations of hematoxylin and eosin. In a recent survey[1] (1945), out of 203 hospital laboratories asked, 161 used forms of hematoxylin ranging through Erlich's acid hematoxylin, Weigert's iron formula, Bohmer's, and Delafield's; 158 made use of picric, aqueous or alcoholic eosin and almost without exception used these with some modification of hematoxylin; only 8 used phloxine, combining it in most cases in the Mallory methylene blue formula (Conn, 1940, p. 155). The author has slides twenty years old in which the hematoxylin-eosin combination had been used and the histologic details are still evident. It remains true, however, that balsam has so faded the eosin that fine details are not visible and the imagination must be used in piecing together the finer cytology. A similar trouble occurs even in new sections, and occasionally slides two or three years old will begin to fade, depending upon the dye and mounting media used. It is the purpose of this paper to discuss the relative advantages of phloxine and then present a suitable technic for its routine histologic use.

Phloxine (Syn: erythrosin BB or B extra; new pink) is a member of the xanthene series and may be either a dichlor or tetrachlor tetrabrom fluorescein. It is a higher homolog of eosin and erythrosin, having none of the orange tinge noticeable in the true eosins and less fluorescence. Its Colour Index No. is 774, and it has an absorption

[1]See: Autotechnicon Methodology (The Technicon Co, New York, 1945) and Whitehead (1945)

maximum of 535.7. At 28° C. it is 50.9% soluble in water and 9.02% soluble in alcohol. It is chemically allied to eosin Y by the presence of four bromine atoms (see Fig. 1).

Phloxine found early use as a bacterial stain when Conn and Holmes (1926) made use of dyes of the fluorescein group for staining dried soil films. They showed that 3′, 6′-dichloro-2,4,5,7-tetrabromo fluorescein showed up as a good bacterial stain in pure culture, deeper than eosin and less acid. Later reports showed that additions

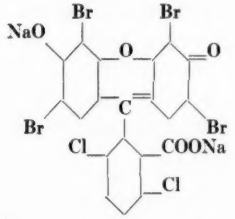


FIG. 1.—The dichlor form of a molecule of tetrabrom fluorescein.

of small amounts of calcium, aluminum, magnesium or lead salts increased the intensity of the stain (Conn and Holmes, 1928). Holmes (1929) formulated a table of solubilities of dyes. The values for phloxine and eosin in these tables are significant.

The stain since has found good use as a bacterial stain (McCalla, 1941; Maneval, 1941).

TABLE 1. RELATIVE SOLUBILITY OF PHLOXINE AND EOSIN AT 26 C°.

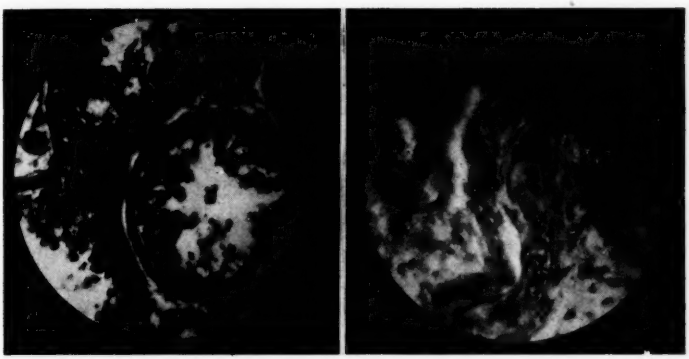
	H_2O	95% alcohol
Phloxine C.I. 774	(Na salt) 50.90	9.02
	(Mg salt) 20.84	29.10
	(Ca salt) 3.57	0.45
	(Ba salt) 6.01	1.17
Eosin C.I. 768	(Na salt) 44.20	2.18
	(Mg salt) 1.43	0.28
	(Ca salt) 0.24	0.09
	(Ba salt) 0.18	0.06

Following World War I, Grübler placed a magdala red on the market which went under the names "magdalarot" and "magdalarot echt". Dr. H. J. Conn in his section on aniline dyes in McClung's "Microscopic Technique" states: "a cheaper magdala red, without qualifying designation, was also put on the market by Grübler before the war, which proves to have been (in some instances at least) a fluorescein derivative (phloxine or erythrosin) rather than a safranin." It was

such a dye that Chamberlain (1924, 1932) found stained algae better than the more expensive brand of magdala red.

Mallory, in his later work, used phloxine in his methylene blue formula. Conn (1925, p. 82) states from personal communication with Mallory that it was found to be the best *eosin* for the technic. The technic was formulated originally in 1903 and was later modified by him, in 1938 to specify phloxine instead of eosin. The formula has been used by the Biological Stain Commission as routine for phloxine samples submitted for certification.

As to variety of use, the dye is extremely flexible. Ballantyne (1940) has reported using phloxine on frozen sections with success. It is adaptable as a botanical stain, having been used successfully by



A B

FIG. 2.—A. Testis. Human × 450; cut at 15 μ and stained by the phloxine-hematoxylin technic. Note the intensity of the stain on the walls of the seminiferous tubules and developing spermatocytes.

B. Uterus. Human × 450; cut at about 10 μ and stained with a standard eosin-hematoxylin schedule. Both sections were fixed in Zenker's fluid. The same light intensity was used in both A and B.

several workers. Buchholz (1938) used phloxine in combination with Delafield's or Heidenhain's hematoxylin on conifer embryos, while Stanley (1940) used the dye in studying certain of the basidiomycetes. In all, phloxine has proven itself most versatile and deserves more recognition in standard routines.

TECHNIC

Very little has appeared in the literature with regard to using phloxine as a counterstain to hematoxylin in tissue work. Margolina

(1933) mentions its use with Heidenhain's or Harris' hematoxylin and orange G as a differential counterstain. The following technic is similar to the standard eosin-hematoxylin technics with modifications in time and reagents:

1. Fix in Zenker's fluid.
2. Embed in paraffin and cut sections at 8 to 10μ for best results. Mount sections on albuminized slides as usual.
3. Place in xylene for 1 minute. Place the sections in two changes of 95% alcohol for 1 minute each. Wash well in tap water. Place in iodine-alcohol for 3 minutes to remove mercuric chloride crystals. To remove the iodine color, place in a weak solution of sodium hypochlorite until the sections are bleached.
4. Place the sections in Erlich's acid hematoxylin for about 7 minutes. Overstaining will not occur, but the 7 minutes prescribed will suffice to show up most nuclei. Wash well in tap water. The solutions will stain better if a good washing is given after each of the above steps.
5. Place in Scotts Bluer ($KHCO_3$,2 g.; $MgSO_4$, 20 g.; distilled water, 1,000 ml.) for ½ minute and wash well. Dip the sections in a 5% solution of acid alcohol and wash quickly in tap water.
6. Place the slides in a 5% solution (aqueous) of phloxine for 10 seconds. The dye is strong and at this strength will give a light but even distribution of pink to the cytoplasm. Wash in tap water thoroughly.
7. Place the slides in 95% alcohol for one minute and drain well. Follow the same procedure for absolute alcohol. Place the slides in a 50% solution of xylene and creosote. This has an intensifying effect upon the stain. The slide may now be placed indefinitely in xylene until the cover slip is applied. Clarite is the recommended mounting medium.
8. Results: Nuclei appear medium purple against a light pink background.

The technic is not too time-consuming, being in most cases shorter than the standard hematoxylin-eosin schedules. It is accurate, resulting in a true differentiation of the necessary histologic elements. Formalin-fixed tissue may be stained with equally good results.

The author expresses his appreciation to Dr. J. W. Denton and Mr. Fritz Carter of the pathological laboratories, New Rochelle Hospital, New Rochelle, N. Y., for introducing him to the value of phloxine as a dye in the above technic.

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CHLORAZOL PAPER BROWN B AS A STAIN FOR PLANT TISSUES

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The staining properties of Chlorazol paper brown B were discovered while a considerable range of Chlorazol dyes from Imperial Chemical Industries Ltd. (Hexagon House, Blackley, Manchester, 9, England) were being investigated with a view to their possible use as selective stains for paper fibers. The dye is not yet available from dealers.

The stain is used in saturated aqueous solution, the solubility being about 30 g. per liter at 14° C. Material may be stained overnight but in many cases, for example where the delicate cell contents are not germain to the investigation, boiling in the aqueous stain for one or two minutes will produce an equivalent depth of staining. The material is then differentiated in 1% nitric acid solution. Any solution of low pH will effect differentiation but nitric acid appears to be the best. Dehydration in acetone and mounting in Euparal have proved to be the most suitable for preserving the colors.

Trials with the stain have not been extensive but the few examples mentioned below will serve to indicate its possibilities. The first material investigated was *Clematis vitalba* L., in transverse section. By employing the boiling technic finished slides could be obtained in about five minutes. The tissues and their respective colors obtained are tabulated below:

Epidermis	Yellow
Cortex	Yellow
Pericycle (lignified tissues)	Blood-Red
Xylem	Salmon to blood-red.
Primary Phloem	Orange
Cambium	Pale yellow
Secondary Phloem	Crimson
Seive Plates	Some very bright crimson, others orange
Pith	Amber

Year old slides of sections of *Clematis* have retained their brilliancy of coloring and no noticeable fading has taken place. Sections of monocotyledenous tissues are not successfully stained by the dye.

Transverse sections of the leaf of *Ananassa sativa* stained in the dye show the epidermis red but the bundles and fibers are scarcely differentiated. Textile fibers are well stained; fine structure is brought out well. Jute, for example, stains very well and the resulting color is admirably suited for photomicrographic purposes.

F. D. Armitage has successfully stained the alga *Spirogyra* with the dye (overnight). He finds, however, that it is of no value for staining fungi, nor does it appear to be of any value for staining animal tissues.

The author wishes to thank the Council of the Printing and Allied Trades Research Association for their permission to publish this note.

A SIMPLE METHOD FOR SQUASHING AND MOUNTING PREPARATIONS AFTER ANY STAIN

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ABSTRACT.—**A method is described for the squashing and permanent mounting of preparations, similar to the nucleal squash technic, to be used after hematoxylin, coal tar dyes and other stains. The colored pieces are immersed in glycerin, squashed or smeared, the glycerin is slowly removed with water by capillarity and the water is afterwards similarly substituted by alcohol. The cover glass is then removed and after a rapid rinsing in alcohol the materials are permanently mounted in alcohol-soluble resin.**

INTRODUCTION

The smearing or squashing method (Nuklealquetschmethode) after a Feulgen reaction (in which the tissues are placed in a few drops of 45% acetic acid and pressed between slide and cover glass until separation of the cells and spreading of the chromosomes is obtained) is largely used in chromosome studies with good results. After smearing, the preparations are either immediately observed, sealing the cover glass with paraffin or other means, or mounted permanently in a suitable resin. The Feulgen reaction only reveals the thymonucleotides, while other nucleal components of proteic, ribonucleic and lipidic nature are not colored. Despite the fact that some combinations with other dyes, for instance with light green, are also used after the Feulgen reaction, the lack of good morphological colorations of the cell elements is felt on many occasions when studying structures without thymonucleic acid.

It should be important to have a procedure equivalent to the nucleal squash method for the rapid mounting of materials colored in hematoxylin or coal tar dyes, etc. Such a method is here described, which we have used for some time in cytological work with good results.

THE GLYCERIN SQUASH TECHNIC

The method consists in staining, after a suitable fixation, small pieces (thickness about 0.5–1 mm.) or freehand sections of the tissues in the desired dye, washing thoroughly, squashing or smearing in glycerin, and mounting in resin. The steps of the procedure are the following:

1. Immerse the colored materials in pure glycerin for about 5 minutes to 2 hours, according to the thickness and the hardness of the pieces, for penetration. If the materials are liable to shrinkage in pure glycerin, use glycerin and distilled water (1:1) or glycerin and 70% alcohol (1:1). Sometimes the glycerin-water (1:1) gives better penetration than pure glycerin.
2. Squash the material by placing it in a drop of pure glycerin on a slide and pressing rapidly but gently between slide and cover glass. Rapid alternating strokes (allowing the cover slip to rise between strokes) give a good separation of the cells.
3. The preparation may be observed in glycerin after sealing with a cement made of lanolin and rosin (2 to 8 parts in weight). Permanent preparations may be easily obtained by mounting in rosin according to steps 4–7.
4. To mount permanently: Immerse the preparation overnight in distilled water in a jar, to about ⅓ to ½ of the cover glass height.
5. After about 12 hours in distilled water immerse the preparation with the cover glass in position (we have never observed a spontaneous detachment of the cover glass in water) in a jar with 96% alcohol. Here also, the level of the alcohol must be about ⅓ to ½ of the cover glass height.
6. About 12–24 or more hours after immersion in alcohol, the cover glass detaches spontaneously, or preferably, is lifted up with the aid of a fine-tipped lancet needle. Usually no loss of material is experienced in this step, the glycerin causing in general a better adherence than acetic acid in the nucleal method.
7. Rinse carefully in 96% alcohol for 3–5 minutes, maintaining the slide and cover glass horizontally, and mount in Euparal or other alcohol-soluble resins.

Notes

If it is desired to mount in xylene soluble resins, it is necessary, of course, to pass through absolute alcohol and xylene, after step 6. It is better, however, to use alcohol-soluble resins, because in successive passages the materials are subject to loss and in many cases the contraction accompanying the treatments with absolute alcohol and xylene considerably diminishes the volume of structures already in the limit of visibility.

The method is applicable to all stains which do not deteriorate in a rapid passage through glycerin. Acetic acid can not be employed

instead of glycerin owing to its action upon hematoxylin and coal tar dyes.

The glycerin squash method can also be applied after a Feulgen reaction, alone or combined with an aqueous counterstaining. It may also be combined with stains dissolved in 96% alcohol, if the stain is applied when step 7 is reached.

The principal advantages of the method are: the saving of time in embedding and sectioning; the possibility of obtaining complete cells not cut by sectioning; and the use of almost any stain before squashing.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

SALMON, M. V. **Practical phase-contrast microscopy.** *The Microscope*, **6**, 177–88. 1947.

The author refers to recent interest in the phase-contrast microscope and explains fairly simply the principles underlying this method of making invisible differences of light phase visible by converting them into visible differences in intensity. He states that the only bar to widespread use of the method has been the difficulty in obtaining the phase discs for use in the objective. The writer describes a method of preparing such a disc, with an etched stripe half a wavelength deep, and of inserting it in an objective. He describes how this should be used in conjunction with the proper slit in the illuminant. Photomicrographs, showing detail brought out by phase-contrast but invisible by ordinary illumination, are given. The paper is of value to microscopists with mechanical ingenuity who are prepared to make such equipment for themselves, rather than to those who depend on manufacturers for all such specialized items.—*H. J. Conn.*

MICROTECHNIC IN GENERAL

EVANS, TITUS C. **Radioautographs in which the tissue is mounted directly on the photographic plate.** *Proc. Soc. Exp. Biol. and Med.*, **64**, 313–15. 1947.

Sections of tissue which contain radioactive material are mounted directly on the emulsion of a photographic plate in the dark room. Paraffin sections are floated on warm water in the usual manner to expand them and then transferred to cool water. The photographic plate, emulsion side up, is slipped under the section. One corner of the section is held against the emulsion while the plate plus the tissue are removed from the water. The preparation is then dried before a fan and placed in a light-tight box. The time required for the radiation period is not stated and it seems likely that it would have to be determined for the type of experimental procedure in use. After radiation, the paraffin is removed from the section with xylene, the preparation dried, and the photographic image developed with Eastman D-11 or D-72 solution. Fixation in acid hypo, washing and drying follow with subsequent staining by hematoxylin and eosin. If it is desirable to use photographic film instead of plates, several methods are suggested for avoiding overstaining of the film. The article is illustrated by three figures.—*H. A. Davenport.*

DYES AND THEIR BIOLOGICAL USES

TOLBERT, B. M., and BRANCH, G. E. K. **The spectra of the doubly charged positive ions of some p,p′-diaminotriphenylmethane dyes.** *J. Amer. Chem. Soc.*, **69**, 1083–91. 1947.

Spectra of the second ions of malachite green and some more highly phenylated diaminotriphenylmethane dyes were measured. Phenylation has a much greater bathochromic effect on the x-band of a second ion than on the corresponding band of a first ion, and the frequency differences between the x-bands and y-bands are greater in second ions than in first ions. Spectra of solutions containing both first and second ions are not linear combinations of the spectra of the first and second ions.—*R. T. Whittenberger.*

VAN WYK, J. J., and CLARK, W. M. **The luminosity and chromaticity of indicators as a function of pH.** *J. Amer. Chem. Soc.*, **69**, 1296–1301. 1947.

The "one-color" indicator, p-nitrophenol, has a dominant wave-length of 568 mμ independent of pH. At the concentration used the indicator exhibits only about 2% change of luminosity between pH 6.1 and 7.7 but a four-fold change of spectral purity. Thus, discrimination between solutions of different pH is based almost exclusively on the change of spectral purity. In contrast, phenol red and brom thymol blue show distinct changes in luminosity, dominant wavelength and purity. The present state of analysis of factors concerned in the discrimination between small color differences does not permit the weighting of the effects of changes in luminosity and changes of chromaticity. However, certain minimal differences in chromaticity are indicated.—*R. T. Whittenberger.*

ANIMAL MICROTECHNIC

HAMRE, CHRISTOPHER J. **Hematopoiesis in the bone marrow of rats recovering from nutritional anemia.** *J. Lab. & Clin. Med.*, **32**, 756–76. 1947.

A study of blood and bone marrow was made in 60 anemic rats during the initial 48 hours of recovery after treatment with copper and iron. Hemoglobin and erythrocytes began to increase within 26 hours. Normoblasts, eosinophiles and heterophiles increased at 12 hours and continued high up to 26 to 28 hours. The first histologic response in the bone marrow was the development of heteroplastic erythropoietic tissue, including numerous pronormoblasts. This was greatest at 18 to 26 hours. At the same period there was great increase in normoblasts, eosinophiles and heterophiles, and these were discharged into the blood stream. This was followed by a period of homoplastic development. There was no rupture or destruction of the lining membrane of the sinuses during discharge of cells from the sinusoidal tissue into the sinuses. "Primitive cell" progenitors of granulocytes were not recognized in the preparations.—*John T. Myers.*

LIMARZI, LOUIS R. **Evaluation of bone marrow concentration techniques. A modified method for the simultaneous preparation and staining of blood and bone marrow films.** *J. Lab. & Clin. Med.*, **32**, 732–40. 1947.

The author proposes the following technic: Under local procaine anaesthesia, introduce a 16 gauge sternal puncture needle at the second interspace, remove exactly 1.0 ml. of sternal fluid and place it in a paraffin-lined tube containing a minute amount of heparin. Mix, transfer to a Wintrobe hematocrit tube and centrifuge 5 minutes at 2000 r.p.m. Remove the fat and most of the plasma. Place one drop of the concentrated cells on a slide, draw a second slide at an angle of 30 to 45° against the drop until it spreads out in the angle, drop the top slide to "sandwich" the drop and remove the top slide. At the opposite end of the slide prepare a similar film with blood from finger or ear. Air dry and stain with Wright's stain. This method avoids dilution of the marrow fluid with peripheral blood and is simple and accurate.—*John T. Myers.*

HEATH, O. V. S. **Role of starch in light-induced stomatal movement, and a new reagent for staining stomatal starch.** *Nature*, **159**, 647–8. 1947.

A reagent which satisfactorily stains leaf starch without causing stomatal closure is prepared as follows: dissolve 2.5 g. of iodine in 100 ml. of phenol, containing the minimum amount of water to make it liquid at room temperature and an excess of KI. This reagent has a very high refractive index, and excellent images may be obtained, either for visual work or photomicrography, by mounting the material in it.—*C. Randall.*

MICROÖRGANISMS

COHEN, H. A. **A new quick method for staining Treponema pallidum.** *Acta Med. Orientalia*, **6**, 99–100. 1947.

This staining method, which takes but 1 min., is based chemically on the reduction of $AgNO_3$ in an alkaline solution. The technic described is the following: (1) A thin film is essential and has, moreover, to be fixed immediately; (2)

Fixation is accomplished by a 36–40% formalin solution with subsequent heating until evaporation occurs. This is followed by flushing with distilled water; (3) Staining: 4 drops of a solution consisting of equal parts of $N/1$ NaOH and $N/10$ NaOH, are added followed by 2 drops of a 5% $FeCl_3$ solution, 4 drops of $N/1$ $NH_2OH \cdot HCl$ (hydroxylamine hydrochloride) and 3 drops of a 10% solution of pyrogallic acid. The slide is then heated until bubbles appear under simultaneous gentle stirring. This is followed by flushing with distilled water, after which a few drops of a 0.5–5% ammoniacal solution of $AgNO_3$ are added. The slide is then washed and dried. After staining, according to this method, Treponema cells assume a reddish-brown color.—*Jas.* (Courtesy *Biological Abstracts*).

HENRY, H., and STACEY, M. **Histochemistry of the Gram-staining reaction for micro-organisms.** *Proc. Roy. Soc., Ser. B. Biol. Sci.*, **133**, 391–406. 1946.

Extraction of *Clostridium welchii* cells with 2% bile salt in normal saline at 60°C. for 12 to 144 hr. extracts one-fourth of the dry weight, leaving a residue of Gram-negative cytoskeletons containing large amounts of a basic protein. Magnesium ribonucleate, isolated from the bile salt extract or prepared from commercial yeast nucleic acid, could be recombined in from 20 min. to several hours with Gram-negative cytoskeletons that were rendered receptive by 18 to 48 hr. treatment with 1% solution of reducing agents such as Na_2SO_3, glyoxal, $NaHSO_3$, ascorbic acid, dihydroxymaleic acid, thioglycollic acid or formaldehyde. Following recombination with magnesium ribonucleate, the cytoskeletons regained their Gram-positive reaction. Gram-stainable material in normal and reconstituted cells appears as opaque granules in ultraviolet photomicrographs (265 mμ) corresponding to the strong absorption band of magnesium ribonucleate at 260 mμ. Bile salt extraction of *Saccharomyees cerevisiae* removed one-tenth of the dry weight, producing Gram-negative cytoskeletons which could be reconverted to Gram-positive by reduction and treatment with magnesium ribonucleate prepared from either yeast, *Clostridium welchii*, or *Bacillus subtilis*. Similar results obtained with *B. oedematiens*, *B. sporogenes*, *Vibrio septicum*, *B. anthracis*, *B. mycoides*, *B. mesentericum*, *B. megatherium*, and certain strains of streptococci and sarcinae are not reported in detail.—*E. H. Shaw, Jr.* (Courtesy *Biological Abstracts*).

PARA, M. **Silver impregnation of spirochetes in tissue sections.** *Arch. Path.*, **42**, 649. 1946.

A new silver impregnation technic is described, which is applied in two possible procedures, the basic and rapid methods. Tissues fixed in a 1:10 dilution in saline of U.S.P. formaldehyde and paraffin sections 4–6 μ thick are used.

The basic procedure is as follows: Immerse deparaffined and hydrated sections 30 min. at room temperature in 1% $UO_2(NO_3)_2 \cdot 6H_2O$, (or 45 min. at 56°C. in 4% aqueous ferrous ammonium sulfate, or 10–15 min. in a sulfur water mixture made up by adding 10 ml. 5% anhydrous $NaHSO_3$ and 10 ml. N HCl to 200 ml. distilled water in a tightly stoppered bottle, or 20 min. at 56°C. in 1% oxalic acid solution, or 30 min. at room temperature in 0.5% $CuSO_4$ or 5 min. at room temperature in 0.02% K_2MnO_4). Wash rapidly in distilled water. Treat 2 hr. at 56°C. with 1.5% aqueous $AgNO_3$ (stored in the dark), replacing the silver solution after the first hour with fresh solution, or overnight at 37°C. with the same silver solution. Wash rapidly in distilled water. Treat 1 hr. at room temperature with the following, made up just before use: 5 ml. 5% rosin (colophony) in ethanol are added with gentle shaking to 45 ml. of a solution prepared by adding 2.0 ml. saturated aqueous Li_2CO_3 (about 1.3%) to 100 ml. boiling 0.2% aqueous $AgNO_3$, boiling 1–2 minutes and filtering through paper (this solution will keep one month in the refrigerator), (or 1 hr. at room temperature with colloidal silver tartrate solution made up by adding 10 ml. 5% alcoholic rosin to 40 ml. of Steiner's double tartrate-silver-nitrate solution, or 1 hr. at room temperature in a mixture of 10 ml. of 5% alcoholic rosin and 40 ml. of 10% aqueous dilution of ox bile, followed by 2 hr. at 56°C. in 1.5% aqueous $AgNO_3$). Immerse 10–15 min. at room temperature in Levaditi's reducing solution consisting of 4% aqueous pyrogallic acid with 5% by volume of USP formalin. Wash rapidly in distilled water. (If microscopic examination at this time shows improper tissue impregnation, treat section rapidly with absolute ethanol, im-

merse 10 min. in the $AgNO_3$-rosin mixture and 10 min. in Levaditi's reducing solution.) Dehydrate, and mount in Canada balsam.

Sections impregnated with the above technic are yellow-brown in color and show brown cell nuclei and black spirochaetes. If the tissue is not properly fixed a poor silver impregnation results. In such a case, cut new paraffin sections from the block; mount, remove paraffin and hydrate to the 50% ethanol stage; treat 1 hr. at room temperature with 2% alcoholic rosin solution; wash in absolute ethanol; rehydrate; and impregnate as previously described.

The following substances may be substituted for the rosin in the sensitizing mixture: gum mastic (add 10 ml. of 2.5% absolute ethanolic gum mastic solution to 40 ml. of $AgNO_3$-Li_2CO_3 solution or Steiner's silver tartrate solution); ox bile (add 5 ml. of fresh ox bile to 4.5 ml. of $AgNO_3$-Li_2CO_3 solution or Steiner's silver tartrate solution); balsam of tolu (add 5 ml. 1% ethanolic balsam of tolu solution to 45 ml. of $AgNO_3$-Li_2CO_3 solution). A solution of 1 g. hydroquinone in 60 ml. distilled water may be substituted for the pyrogallic-acid-formaldehyde reducing solution.

The procedure for the rapid method is as follows: Hydrate sections after removal of paraffin; treat 1 hr. at 56°C. with a freshly mixed preparation containing 10 ml. of 1% aqueous $AgNO_3$, 10 ml. of a dilution of 1 ml. of 1.3% Li_2CO_3 in 165 ml. of distilled water (or 10 ml. of 0.1% sodium potassium tartrate solution) and 10 ml. of 2% ethanolic rosin solution (dilute filtered 50% rosin 25 times with absolute ethanol just before use); treat 7–10 min. at room temperature in a reducing mixture consisting of 50 ml. of Levaditi's reducing solution and 5 ml. of 2% ethanolic rosin solution; wash 5 min. in each of 2 or 3 baths of 90% ethanol; wash rapidly in distilled water; replace 15 min. at 56°C. in the $AgNO_3$-Li_2CO_3-rosin mixture; treat 3–5 min. in the above reducing mixture; wash 2 min. in each of 2 baths of 90% ethanol; dehydrate and mount in Canada balsam.

A combined process for studying pathological changes in the preparations as well as demonstrating spirochaetes is also described. This is as follows: Take tissue section through the rapid impregnation procedure up to the dehydration step, except that no rosin is used in the reducing solution; wash in distilled water; stain 10–15 min. in Harris' hematoxylin; pass rapidly through the dilution of 1 ml. 1.3% Li_2CO_3 in 165 ml. distilled water; wash in running water; stain 1–3 min. in Altmann's acid fuchsin diluted 5 times in distilled water before use; differentiate in absolute ethanol for 5 min.; dehydrate and mount in Canada balsam.

With the above technic the cytoplasm is stained light red, the nuclei dark violet, the connective tissues bright red or ochre yellow and the spirochaetes black.

Von Gieson's stain for 1 min. may be used in place of Altmann's acid fuchsin. Meyrick and Harrison's stain (15 vol. 1% aqueous neutral red and 1 vol. carbol fuchsin made by adding 9 ml. 10% alcoholic basic fuchsin to 90 ml. 5% aqueous phenol) for 10 min. may be substituted for the hematoxylin stain followed by rapid differentiation in absolute ethanol, dehydration and mounting in Canada balsam. With this latter staining modification the cytoplasm is stained light red, the nuclei bright red, the connective tissues dark red or greenish yellow and the spirochaetes black.—*L. Farber.*

RUIZ, MERINO, J. **A method of staining capsules.** *Rev. Sanidad e Hig. Publ.* (Madrid), 20, 1112. 1946.

This easy method requires the bacteria to be growing under conditions to obtain the largest development of their capsules. The reagents used are: Solution A, methyl violet, 1%; Solution B, yellowish eosin, 0.01 g., distilled water, 10 ml., horse normal serum, 3 ml.

One drop of reagent A is mixed with the material to be stained using a platinum needle and left 5 min. in a moist room to avoid evaporation; then one drop of solution B is added, mixing it for 3 min., and with a glass slip a smear like those of hematology is made; it is observed with a microscope after drying. —*I. Pemander.* (Courtesy *Biological Abstracts*).

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